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Genotypic Identification of Extended-Spectrum β-Lactamase (ESBL)-Producing Enterobacteriaceae from Urinary Tract Infections in the Leicestershire Area, United Kingdom: A One Health Prospective

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Abstract

Objectives: Urinary Tract Infections (UTIs) are one of the most common infections diagnosed in the United Kingdom (UK). The prevalence of Extended-Spectrum-B-Lactamase (ESBL) producing UTIs has dramatically risen, limiting treatment options. The emergence and spread of ESBLs is thought to be through the horizontal transmission of antibiotic resistance plasmids IncL/M, IncF, IncN and IncI1. These conjugative plasmids have been described as important vectors and directly linked to major outbreaks of antibiotic resistance. This study aimed to investigate the prevalence of ESBLs in Leicestershire, UK and their relationship with antibiotic resistance plasmids.

Methods: 236 ESBL producing uropathogenic Enterobacteriaceae isolates were obtained from the Leicester Royal Infirmary (Leicestershire, UK). ESBL production was confirmed phenotypically *via* the MAST ID double disc synergy test. ESBL-producing genes (*CTX-M*, *SHV*, *TEM* and *OXA*) were identified by multiplex PCR. The *CTX-M* family was then further characterised into (*CTX-M-1*, *CTX-M-2*, *CTX-M-8*, *CTX-M-9* and *CTX-M-25*) by multiplex PCR. The relationship between ESBL-producing genes and plasmid type was then investigated by multiplex PCR-based replicon typing to detect IncFIA, IncI1, IncL/M, IncN and IncFII.

Results: ESBL genes were identified as follows: *CTX-M* (71.6%), *OXA* (7.6%), *TEM* (3.8%) and *SHV* (3.8%). Multiple resistance genes were detected in 16% of isolates. *CTX-M* genes were identified as follows: *CTX-M-1* (84.1%), *CTX-M-9* (12.5%), *CTX-M-25* (1.7%), *CTX-M-8* (1.1%) and *CTX-M-2* (0.6%). Replicon typing results were as follows: IncL/M (29.2%), IncN (14.4%), IncI1 (5.1%), IncFII (27.5%) and IncFIA (23.3%). A combination of IncL/M, IncFII and IncFIA was the most common at 9.8%. A positive correlation between *CTX-M* and all plasmids except IncI1 was found.

Conclusion: *CTX-M* harbouring Enterobacteriaceae are associated with multiple plasmids, which can be linked to its rapid spread across the world. Prevalence studies help to inform policy about antibiotic stewardship and resistance evolution, aiming to reduce resistance levels in the future.

Keywords: Extended-spectrum-β-lactamases; Multiplex PCR; Antibiotic resistance; *CTX-M*; Urinary tract infection; Replicon typing; One health approach

Introduction

Urinary Tract Infections (UTIs) are one of the most common bacterial infectious diseases diagnosed in outpatients in the UK. Of the causes of UTIs, Uropathogenic Escherichia coli (UPEC) are the most prevalent [1]. Risk factors for acquiring a UTI include previous exposure to 3rd and 4th generation cephalosporins and fluroquinolones, hospitalisation, old age, female gender, recurrent UTI infection, diabetes and catheterisation [1].

The most common type of resistance in UTIs is β-lactamase production, resulting in extended-spectrum β-lactamase (ESBL) producing bacteria [2]. These bacteria produce enzymes that can hydrolyse oxyimino-cephalosporins (ceftriaxone, cefotaxime, ceftazidime and cefepime) and monobactams (aztreonam) but not cephamycins and carbapenems. Based on their amino acid sequence, these enzymes have been classed into four groups-A-D [2]. Class A enzymes have been shown to be the most clinically important, due to their link to treatment failure, increased morbidity, mortality and healthcare costs. In contrast to the class A, C and D serine β-lactamases, class B are metallo-β-lactamases. Class C β-lactamases are AmpCs, conferring resistance to not only third-generation cephalosporins but also β-lactam/β-lactamase inhibitor combinations. The most important class D β-lactamase is the *OXA*-type. There are 3 main families of class A: *TEM*, *SHV* and *CTX-M* while *TEM*, *SHV* and *OXA* ESBLs arise *via* substitutions in strategically positioned amino acids from the natural narrow-spectrum *TEM*-1/-2, *SHV*-1 and *OXA*-10 β-lactamases, all *CTX-M* variants demonstrate an ESBL phenotype [2]. Previously, most ESBLs detected were of the *TEM*/*SHV* group. From the 1990's the *CTX-M* family became increasingly more common, and now it is reported to be the most prevalent type of ESBL detected at present worldwide [2]. The worldwide dissemination and dramatically increasing prevalence of the *CTX-M* family of ESBLs is due to the selective pressure caused by the over-use, and more importantly, mis-use of the β lactam antibiotics [2].

Risk factors for an ESBL-producing UTI are; recent hospitalisation, treatment with 3rd generation Cephalosporins, old age (over 65), diabetes, recurrent UTI, indwelling catheters and female gender [3]. It has been reported that ESBL-producing isolates are more likely to be associated with significant pyuria, suggesting that ESBLs are more likely to cause a clinically significant UTI [4,5]. Although most ESBL-producing UTIs are acquired in the community, a hospitalised patient is approximately 4 times more likely to be diagnosed with an ESBL-producing UTI [3].

The significant increase in movement of livestock and agricultural produce and human travel has facilitated the rapid amalgamation and dissemination of antibiotic resistance genes [6]. The emergence and spread of antibiotic resistance determinants is often through horizontal transmission of mobile genetic elements such as plasmids. [7]

It is the large, low-copy, self-transmitting resistance conjugative plasmids that are increasingly threatening the efficacy of antibiotics for Gram-negative infections and have been directly linked to antibiotic resistance outbreaks. Conjugative plasmids can carry more than one gene for a selectable advantage such as antibiotic resistance genes and virulence factors such as bacteriocins and cytotoxins. Under antibiotic and infectious pressure, these traits may facilitate the successful spread of certain plasmid types between different bacterial hosts and geographical locations [8]. This results in ESBL-producing isolates that can show resistance to other types of antibiotics, leading to multidrug resistance (MDR). MDR limits treatment options, resulting in less favourable outcomes for patients [9]. Surveillance of the spread of these plasmids can help track antibiotic resistance determinants and phylogenetic and comparative analysis can identify the origin and evolution of these genes [10].

Many papers have discussed the importance of plasmids in the spread of bla*CTX-M* genes, especially *CTX-M-1* [11-16]. Studies have suggested that *CTX-M* is primarily associated with plasmids of IncF, IncL/M, IncN and IncI1 and these have been termed as "epidemic resistance plasmids" in Enterobacteriaceae [11,15,17,18]. It is suggested that these plasmids are successful as they likely provide virulence and antimicrobial resistance determinants, such as ESBL-production, that contributes to the fitness of the bacterial host. They also guarantee their survival and stability in the host through the production of several addiction systems, such as toxin-antitoxin systems. Therefore, they are maintained without the need for selective pressure of antibiotic treatment. This could explain the rapid dissemination of *CTX-M* genes within Enterobacteriaceae [19-21].

Realising the major health concern that antibiotic resistance presents, the world health organisation (WHO) developed a global strategy for the containment of antimicrobial resistance in 2001. This involved monitoring the prevalence of ESBLs in the UK, and other countries all over the world. Prevalence studies help to inform policy on infection prevention and antibiotic stewardship. They allow monitoring of evolution of antibiotic resistance genes and prediction of possible future evolution [22]. The importance of surveillance was reiterated in the O'Neill report where it was suggested that all antibiotic prescriptions should be informed by up-to-date surveillance information [23-25].

Prevalence studies are an important way of monitoring the transmission of resistance determinants between vectors, and whether stewardship and other measures are working effectively [23]. The aim of this study is to identify the prevalence of the different ESBL types in Leicestershire by multiplex PCR. PCR-based replicon typing will be used to investigate whether prevalence is associated with antibiotic resistance plasmids. We hypothesise that the prevalence of ESBLs in this area is related to the conjugative resistance plasmids IncL/M, IncF, IncN and IncI1.

Materials and Methods

Isolate collection and phenotypic detection

Bacterial isolates (n=236) of Enterobacteriaceae isolated from urinary tract infections were obtained from the Leicester Royal Infirmary hospital (Leicester, England). The Leicester Royal Infirmary was chosen as a collection site as a large number of samples are received from all over the Midlands and the Midlands has a large population of a wide variety of ethnic groups. According to the 2011 Census, 20.8% of people in the West Midlands and 14.6% of people in the East Midlands identify as an ethnicity other than white British [26]. Specifically in the city of Leicester, 49.5% of people identify as non-white British, of this 37.1% identify as Asian/Asian British [27]. ESBL production was confirmed phenotypically *via* the MAST ESBL ID double disc synergy method, conforming to British Society of Antimicrobial Chemotherapy (BSAC) standards [28]. Discs contained Cefotaxime, Ceftazidime and Cefpodoxime with an ESBL inhibitor (Clavulanic acid) counterpart. Plates were incubated for 18 hours at 37°C. A zone of inhibition difference of 5 mm or more between the β-lactam and the ESBL inhibitor clavulanic acid indicated a positive result for ESBL production. Four control isolates were obtained from Public Health England: NCTC 13353 (*CTX-M-15*), NCTC 13351 (*TEM*-3), NCTC 13368 (*SHV*-18) and NCTC 13442 (*OXA*-48) for use as controls in phenotypic and genotypic tests.

Ethical approval

Ethical approval for this study was not required as Informed consent from patients was not required, as we collected only waste material (bacterial cultures), and therefore there is no link to patient data.

DNA extraction and genotypic detection by multiplex PCR

We have previously outlined the method used for DNA extraction [29]. Genotypic identification was by means of a multiplex PCR method for the detection of the ESBL-producing genes (*CTX-M*, *SHV*, *TEM* and *OXA*). These were then further characterised into the *CTX-M* family (*CTX-M-1*, *CTX-M-2*, *CTX-M-8*, *CTX-M-9* and *CTX-M-25*) by adapting a multiplex PCR assay by Al-Mahayie [30]. The GoTaq G2 Flexi DNA Polymerase kit was chosen for all PCR assays (Promega,Southampton, UK). PCR amplification reactions were performed using the PikoReal® 96 well RT-PCR platform (ThermoFisher, Loughborough, UK) in a volume of 25 µl containing 5 µl buffer, 2 µl MgCl2, 0.5 µl DNTPs, 0.125 µl Taq polymerase, 400 nM of each primer, 2.5 µl of DNA template and made up to 25 µl with water.

Cycling parameters for the first multiplex assay were as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at 65°C for 1 min (gradual temperature decrements of 0.5°C per cycle, final annealing temperature 48°C), and 72°C for 1 min; and with a final extension at 72°C for 10 min.

Cycling parameter for the second multiplex assay were as follows: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 25 s; 52°C

for 40s and 72°C for 50 s; and a final extension at 72°C for 6 min. Primers can be found in Table 1.

Primer	Forward Primer	Reverse Primer	References
TEM-1	CGG ATG GCA TGA CAG TAA GAG	AGG ACC ACT TCT GCG CTC G	[29]
SHV-18	CTCAAGGATGTATTGTGGTTATGC	CTA CGA GCC GGA TAA CGC G	[29]
CTX-M	CGTCATCTATGTTCGCCGAC	GCATCTCAGTCGGATCGAGC	[29]
OXA-48	CGGAATGCCTGCGGTAGCAAAG	CAGCCCTAAACCATCCGATG	[29]
CTX-M-1	AAA AAT CAC TGC GCC AGT TC	AGC TTA TTC ATC GCC ACG TT	[30]
CTX-M-2	AGC TTA TTC ATC GCC ACG TT	CCA GCG TCA GAT TTT TCA GG	[30]
CTX-M-8	TCG CGT TAA GCG GAT GAT GC	CAA AGA GAG TGC AAC GGA TG	[30]
CTX-M-9	CAA AGA GAG TGC AAC GGA TG	ATT GGA AAG CGT TCA TCA CC	[30]
CTX-M-25	GCA CGA TGA CAT TCG GG	AAC CCA CGA TGT GGG TAG C	[30]

Table 1: Primers used in this study. Primers for the first multiplex PCR assay were *TEM*, *SHV*, *CTX-M* and *OXA*. The primers for the second multiplex PCR assay were *CTX-M-1*, *CTX-M-2*, *CTX-M-8*, *CTX-M-9* and *CTX-M-25*. Primers were designed using the OligoAnalyzer 3.1 (Integrated DNA Technologies Inc, Illinois, USA) Primers were then checked for specificity by the BLAST software (National Center for Biotechnology Information, Bethesda, USA).All PCR products were ran on a 2% agarose gel (UltraPure™ Agarose, ThermoFisher Scientific, Paisley, UK) for 90 mins at 60 Volts. A GeneRuler 50 bp DNA Ladder, ready-to-use (ThermoFisher Scientific, Paisley, UK) was used for comparison.

Multiplex PCR-based replicon typing

Each isolate was sub-cultured in Luria Bni broth overnight at 37°C. Plasmids were extracted using the Illustra PlasmidPrep Mini Spin Kit (GE Healthcare Life Sciences, Buckinghamshire, UK) Detection of plasmids was by means of adapting the replicon typing assay designed by [31]. PCR amplifications were performed in a volume of 25ul containing 5 µl buffer, 2 µl magnesium chloride, 0.5 µl DNTPs, 0.125 µl Taq polymerase, 400 nM of each primer and 2.5 µl of plasmid DNA. Cycling parameters were as follows: initial denaturation at 94°C for 5 min 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 5 min. Primers can be found below in Table 2.

Statistical analysis

The Chi Square test was used for statistical comparison and the Spearman Rank Correlation test was used to analyse the relationship between plasmids and ESBLs. P values < 0.05 were regarded as significant.

Primer	Forward Primer	Reverse Primer	Reference
IncFII	TGTTTCCACTATGACCCGCT	TGATACATCGAGGGCAGCAA	This study
IncI1	CGGGAATGTCTGTTGTTGCA	ATCGGCTTCATCCTGGTGAA	This study
IncFIA	CCA TGC TGG TTC TAG AGAAGGTG	GTATATCCTTACTGGCTTCCGCAG	[31]
IncN	GTCTAACGAGCTTACCGAAG	GTTTCAACTCTGCCAAGTTC	[31]
IncL/M	GGATGAAAACTATCAGCATCTGAAG	CTGCAGGGGCGATTCTTTAGG	[31]

Table 2: Primers used in multiplex replicon-typing. Primers for IncFII, IncI1, IncFIA, IncN and IncL/M were designed using the OligoAnalyzer 3.1 (Integrated DNA Technologies Inc, Illinois, USA) Primers were then checked for specificity by the BLAST software (National Center for Biotechnology Information, Bethesda, USA). All PCR products were ran on a 2% agarose gel (UltraPure™ Agarose, ThermoFisher Scientific, Paisley, UK) for 90 mins at 60 Volts. A GeneRuler 50 bp DNA Ladder, ready-to-use (ThermoFisher Scientific, Paisley, UK) was used for comparison.

Results

236 Enterobacteriaceae were tested by multiplex PCR for the ESBL genes *CTX-M*, *TEM*, *SHV* and *OXA*; and the *CTX-M* sub-groups *CTX-M-1*, *CTX-M-2*, *CTX-M-8*, *CTX-M-9* and *CTX-M-25*. Multiplex PCR-based replicon typing tested for IncFII, IncFIA, IncL/M, IncN and IncI1.

The PCR assay assigned the four controls to their correct phylogenetic groups. ESBL genes were identified as follows: *CTX-M*=169 (71.6%) *OXA*=18 (7.6%), *TEM*=9 (3.8%) and *SHV*=9 (3.8%). Multiple resistance genes were detected in 16% of isolates, specifically

CTX-M+*OXA*=16 (6.8%), *CTX-M*+*TEM*=12 (5.1%), *CTX-M*+*OXA* +*SHV*=5 (2.1%) and *CTX-M*+*SHV*=5 (2.1%). This can be seen in Table 1. 31 isolates did not contain any of the four genes tested.

CTX-M genes were identified as follows: *CTX-M-1*=148 (84.1%), *CTX-M-9*=22 (12.5%), *CTX-M-25*=3 (1.7%), *CTX-M-8*=2 (1.1%) and *CTX-M-2*=1 (0.6%). This can be seen in Table 2. All isolates identified as containing *CTX-M* in the previous assay, contained at least one of the *CTX-M* sub-groups tested. One isolate contained both *CTX-M-1* and *CTX-M-8*.

Genes	Number of isolates (n=236)
CTX-M	169
TEM	9
SHV	18
OXA	9
CTX-M + OXA	16
CTX-M + OXA + SHV	5
CTX-M + SHV	5
CTX-M + TEM	12

Table 3: Frequency of ESBL producing UTI isolates in the Leicestershire area. 236 isolates were tested for the presence of *CTX-M*, *TEM*, *OXA* and *SHV* genes by multiplex PCR analysis. All genes were detected, however *CTX-M* was the most frequent.

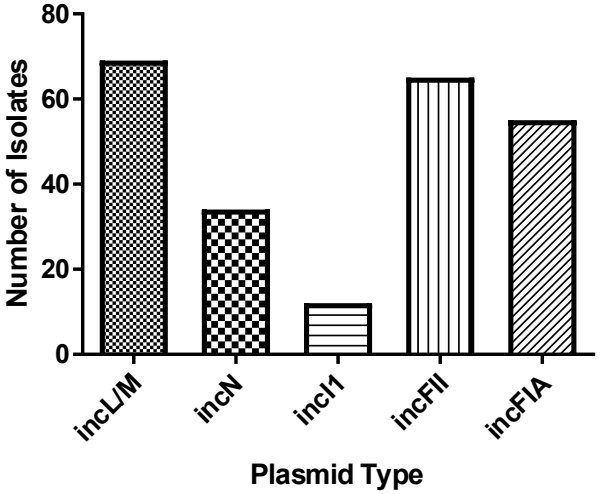

Figure 1: Frequency of replicon types in ESBL producing UTI isolates in the Leicestershire area. 236 isolates were tested for the presence of IncL/M, IncN, IncI1, IncFII and IncFIA genes by multiplex replicon-typing PCR analysis. All genes were detected, however IncL/M was the most frequent, followed closely by IncFII.

Plasmids identified were as follows: IncL/M was detected in 69 (29.2%) of isolates, IncFII was detected in 65 (27.5%) of isolates, IncN was detected in 34 (14.4%) of isolates, IncFIA was detected in 55 (23.3%) of isolates and IncI1 was detected in 12 (5.1%) of isolates. This can be found in Figure 1. 235 of the 236 isolates contained at least one plasmid (Table 3).

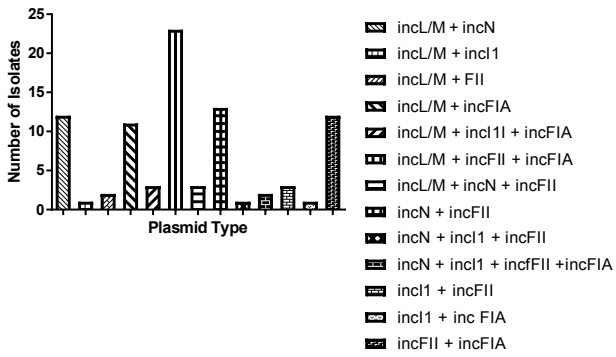

Figure 2: Frequency of dual, triple and quadruple plasmids in ESBL producing UTI isolates in the Leicestershire area. 236 isolates were tested for the presence of IncL/M, IncN, IncI1, IncFII and IncFIA genes by multiplex PCR analysis. All genes were detected, however *CTX-M* was the most frequent.

Multiple plasmids were found in 37% of isolates. . IncL/M, IncFII +IncFIA was detected in 23 (9.8%) isolates. IncN+IncFII was detected in 13 (5.5%) isolates. IncL/M+IncN was detected in 12 (5.1%) isolates. IncFII+IncFIA was detected in 12 (5.1%) isolates. IncL/M+IncFIA was detected in 11 (4.7%) isolates. IncL/M, IncI1+IncFIA was detected in 3 (1.3%) isolates IncL/M, IncN and IncFII was detected in 3 (1.3%) isolates. IncI1 and IncFII was detected in 3 (1.3%) isolates. IncN, IncI1, IncFII+IncFIA was detected in 2 (0.9%) isolates. IncL/M+IncFII was detected in 2 (0.9%) isolates (Figure 2). IncL/M+IncI1 was detected in 1 (0.4%) isolate. IncN, IncI1+IncFII was detected in 1 (0.4%) isolate. IncI1+IncFIA was detected in 1 (0.4%) isolate. This can be found in Table 4.

Genes	Number of Isolates (n=169)
CTX-M-1	148
CTX-M-2	1
CTX-M-8	2
CTX-M-9	22
CTX-M-25	3
CTX-M-1 + CTX-M-8	1

Table 4: Frequency of *CTX-M* sub-groups in UTI isolates in the Leicestershire area. 169 isolates previously identified as containing the *CTX-M* gene, were tested for the presence of *CTX-M-1*, *CTX-M-2*, *CTX-M-8*, *CTX-M-9* and *CTX-M-25* genes by multiplex PCR analysis. All genes were detected, however *CTX-M-1* was the most frequent. A combination of *CTX-M-1* and *CTX-M-8* was found.

A spearman rank was carried out to access the correlation between ESBL genes and plasmid type. Results can be found in Table 5. A significant positive correlation between the *CTX-M* gene and IncL/M, N, FII and FIA plasmids was found. Also found was a significant

positive correlation between the IncFII plasmid and the *CTX-M-8* gene. None of the other *CTX-M* sub-types (*CTX-M-1*, *CTX-M-2*, *CTX-M-9* and *CTX-M-25*) had a significant correlation with any of the plasmids. No significant correlation was found for the *TEM*, *SHV* and *OXA* genes and any of the plasmids.

	CTX-M	***TEM***	***OXA***	***SHV***	***CTX-M-1***	***CTX-M-2***	***CTX-M-8***	***CTX-M-9***	***CTX-M-25***
IncL/M	0.200 (0.002)	-0.033 (0.619)	0.094 (0.1489)	0.010 (0.873)	0.028 (0.674)	-0.042 (0.519)	0.042 (0.525)	0.013 (0.846)	-0.074 (0.262)
IncN	0.148 (0.023)	0.026 (0.694)	0.097 (0.1373)	0.007 (0.918)	0.080 (0.220)	-0.027 (0.681)	0.093 (0.154)	-0.081 (0.214)	-0.047 (0.474)
IncI1	0.029 (0.660)	-0.067 (0.303)	-0.036 (0.5781)	0.105 (0.109)	0.034 (0.604)	-0.016 (0.809)	-0.023 (0.732)	0.079 (0.231)	-0.028 (0.674)
IncFII	0.168 (0.010)	-0.030 (0.646)	0.042 (0.5224)	-0.028 (0.664)	0.016 (0.811)	-0.041 (0.528)	0.147 (0.025)	-0.037 (0.569)	-0.072 (0.271)
IncFIA	0.170 (0.009)	-0.040 (0.543)	-0.004 (0.9568)	0.127 (0.051)	0.055 (0.401)	-0.037 (0.576)	0.057 (0.388)	-0.059 (0.369)	-0.064 (0.330)

Table 5: Correlation coefficients and associated level of significance for the establishing the association between the plasmids and the ESBL genes *CTX-M*, *TEM*, *OXA*, *SHV*, *CTX-M-1*, *CTX-M-2*, *CTX-M-8*, *CTX-M-9* and *CTX-M-25*. Correlation was determined using Spearman Rank analysis. A value 1=perfect correlation; 0-1=no correlation; 0--1=one variable increases as the other decreases; -1-0= perfect inverse correlation. P values are given in brackets and P values <0.05 were regarded as significant.

Discussion

The major cause of antibiotic resistance in Enterobacteriaceae is ESBL-production. ESBL-producing UTIs are a major problem in the UK, due to their increasing prevalence and limited treatment options [32]. Given how common ESBL-producing infections are worldwide, prevalence studies are important to ascertain where resistance is a major problem.

In this paper, the prevalence of ESBL genes was investigated in UTIs in the Leicestershire area of the UK. In short, the *CTX-M* gene was found in 71.6% of samples, in comparison genes *TEM*, *SHV* and *OXA* were detected in ≤ 16% of samples. The most prevalent *CTX-M* was *CTX-M-1* at 84.1%. The most common plasmid found was IncL/M at 29.2%, with IncFII following closely at 27.5%. Multiple plasmids were found in 37% of isolates, with the IncL/M, IncFII and IncFIA combination the most common. A significant correlation between *CTX-M* and all plasmids except IncI1and more specifically, *CTX-M-8* and IncFII was found, however no other gene family significantly correlated with any of the plasmids.

Here, the relevance of our findings to other studies will be evaluated within the context of the One Health Approach. The One Health Approach describes a holistic and multisectoral approach to antimicrobial resistance, as resistant organisms exist in humans, animals, food and the environment [33]. The main aim of the One Health Approach is to ensure that antimicrobial agents continue to be effective by developing policies that promote the responsible use of antimicrobial agents. The development of policy decisions depend on economic and scientific evidence. Prevalence studies are an important part of this [34].

As far as we are aware, this is the first study to investigate the prevalence of ESBL-producing Enterobacteriaceae and their relationship to plasmids, in the Leicestershire area of the UK.

In this study, we found that *CTX-M* was the most common ESBL and the most common sub-group was *CTX-M-1*. This is comparable with other European countries, and is consistent with the worldwide dissemination of the *CTX-M* ESBL [9,15,35,36]. *CTX-M-1* has been found to be the most prevalent gene in humans, animals and food in the UK, frequently found in poultry and cattle isolates [37]. It has been established that the prevalence of ESBLs differs significantly between countries. Within Europe, a high prevalence has been seen in Southern Europe, while a generally lower prevalence is seen in Northern Europe. Outside of Europe, Turkey and India have reported high levels of ESBLs [13].

The high number of *CTX-M* isolates found in this area may be due to the multicultural nature of the Leicestershire community. A study in a hospital in Birmingham, UK also found a high level of ESBL and suggested that this could be due to a high immigrant population [5]. One possible cause of elevated levels of ESBLs in high immigrant populations could be faecal colonisation. Transmission of faeces to the urinary tract is frequently a cause of UTI. As E.coli is a common inhabitant of the gastrointestinal tract, it is possible that *CTX-M*-harbouring *E.coli* could be a commensal bacterium that can become pathogenic upon colonization of the urinary tract [38]. To further this, a direct link between conjugative resistance plasmids in the micro flora and increased treatment failure has been found [39]. In another study in Birmingham, they found a direct link between place of birth and *CTX-M-1* colonisation in stool samples. Those that were originally born in Afghanistan saw the highest colonisation at 60%, followed by those born on the Indian subcontinent (India, Pakistan, Bangladesh or Sri Lanka) with a 25% colonisation. Those that travel to certain areas were also associated with *CTX-M* colonisation. Travellers to South Asia (India, Pakistan, Bangladesh, Sri Lanka or Nepal) in the last year had a 38.5% colonisation, suggesting a geographical, rather than ethnic susceptibility [36].

In India, a prevalence rate of 53% for *CTX-M* was seen in a study detecting ESBLs in UTIs. Antibiotic usage in India is far higher than in

the UK, due to issues with lack of restriction in antibiotic prescribing which could explain why they saw a higher incidence of *CTX-M* [40].

Prevalence rates of *CTX-M* have been seen as high as 94.4% in Iran, 73% in Mexico, 98.7% in Japan and 100% in Ghana [36]. These studies show that the problem of ESBL producing infections is not limited to the UK. As bacteria do not recognise geographical borders, so too does our approach to tackling AMR need to be borderless.

We found that 16% of isolates contained more than one ESBL type. Interestingly, in all of the multi-ESBL isolates *CTX-M* was always present, this suggests that *CTX-M* may play a key role in the production of MDR bacteria. Of these, *CTX-M* and *OXA* were the most common multi-ESBL, detected in 16 isolates. 2% of isolates contained three genes, specifically, *CTX-M*, *OXA* and *TEM*. It has been reported that *CTX-M-1*5 combined with an *OXA*-30 gene can survive β-lactamase inhibitors, since *OXA*-30 is poorly inhibited and also confers resistance to cefepime [12]. Although we did not specifically type the ESBL genes in these isolates, it is possible that it could be this combination present. The coexistence of *CTX-M* and other antibiotic resistance genes could be one of the reasons why *CTX-M* has been so successful. This has been found elsewhere, with incidences of combinations of ESBLs as high as 95.4%, with regards to the *CTX-M* and *SHV* combination [41,42]. Our combination of three genes has also been found elsewhere [42]. Furthermore, other Multi ESBL combinations have also been found in animals and the environment, suggesting that multiple ESBL combinations are not limited to humans. This data combined shows that multi ESBLs are widespread and could pose a significant threat [4,43].

A similar incidence of ESBLs has been reported in livestock and companion animals globally [44-47]. In a study looking at canine UPEC in Switzerland, *CTX-M-1* was found in 28.6% of ESBL-producers suggesting that dogs, a common companion animal worldwide, may be a reservoir for *CTX-M-1* spread into humans [47]. With raw meat a speciality in some countries and the rise of raw food diets in both humans and companion animals, a high level of ESBLs in livestock is a cause for concern. Both livestock and companion animals could be considered a major vector of ESBL-producing bacteria [37]. As it has been suggested that the majority of ESBL infections are community-acquired, this could suggest a relationship between companion animals, livestock and humans.

This study focused on the prevalence of IncL/M, IncFII, IncI1, IncFIA and IncN. These conjugative plasmids have previously been associated with the dissemination of antibiotic resistance determinants such as ESBLs, in Enterobacteriaceae and other bacteria.

Whilst IncL/M was the most common plasmid found in the isolates tested, when IncFII and IncFIA (both members of the IncF group) are combined, the frequency is far higher at 50.8%. The IncF group is one of the most common plasmids found in UTI isolates and is frequently associated with *CTX-M* [12]. Studies show that IncL/M is also highly common, and both have been termed as "epidemic" plasmids [11]. Both of these plasmids can persist for months without the need for selective pressure by antibiotic usage [39]. This suggests that simply reducing antibiotics by antibiotic stewardship alone will not reduce the prevalence of these plasmids.

The distribution of plasmids harbouring ESBL genes in this population differs from the distribution described in other studies. Studies have agreed that *CTX-M* is associated with IncN, IncFII and IncL/M, however reports have shown that *CTX-M* is also associated with IncI1. IncI1 plasmids harbouring *CTX-M* have been described in the UK, US, Belgium, Netherlands, France and Australia in isolates from horse, cattle and human isolates [16]. Studies have also found that other ESBL types are associated with particular plasmids. It has been found that *SHV* can be associated with IncFII, IncL/M and IncI1. Likewise, *TEM* can be associated with the same plasmids, and additionally IncI1 [19]. A reason for the differing results could be due to different antibiotic and environmental selection pressures in this population, leading to selection of *CTX-M* harbouring IncL/M, IncF, and IncN plasmids, but not IncI1 plasmids.

In this study, no significant correlation between *CTX-M-1* (the most common *CTX-M* subtype) and any other plasmid was found. There are conflicting reports within the literature with regards to the most significant association between *CTX-M-1* and plasmid types. Some reports suggest a correlation between *CTX-M-1* and IncFII, whilst others, mainly in Europe, suggest a correlation between *CTX-M-1* and IncI1. Other reports suggest no overall correlation at all [37,48]. It appears that the association between *CTX-M-1* and plasmids differs between geographical locations and sample type. These differences in reports could be attributed to socioeconomic factors, animals and number of samples. It could be suggested that *CTX-M-1* is equally adapted to most plasmid types therefore a significant correlation between just one plasmid was not seen. It could also be suggested that the high level of *CTX-M-1* in this population is also due to another mechanism other than conjugative plasmids, such as chromosomally mediated resistance.

A significant correlation between *CTX-M-8* and IncFII was found. Whilst *CTX-M-8* is not the highest prevailing gene found in this study, it is a significant finding, as IncFII was very common. A link has been found between *CTX-M-8* harbouring IncI1 plasmids and companion animals. This could further strengthen the argument that *CTX-M* genes could be passed, via plasmids, from companion animals to humans [46].

Though studies suggest that IncN does not contribute to the prevalence of *CTX-M* in humans to the same extent as incI1, this was not the case in this study, as IncN was seen to be more prevalent than IncI1 and a significant correlation was found [21]. This theory has also been contradicted by another study that found that IncN was in fact associated with humans and 95.5% of isolates containing IncN harboured *CTX-M-1* [37]. In a Danish study, IncN plasmids carrying *CTX-M-1* were seen in both pigs and farm workers, and it was demonstrated that these plasmids were transmitted within the farm, showing animal to human transmission of these plasmids harbouring *CTX-M* [11].

Multi-replicon plasmids were found in 37% of isolates. Multiple plasmids frequently occur in the same bacterial cell, however, cross-interference between plasmid replicons guarantees that the most closely related plasmids are incompatible and cannot stably persist together [39]. When multi-replicon plasmids occur, generally one replicon is usually highly conserved, and the other is free to diverge. This gives the bacterial host the benefit of being able to change antibiotic resistance determinants and virulence factors, depending on antibiotic pressure and environment [16]. Multi-replicons have been found in other UTI isolates, and multi-replicons containing multiple IncF types appear to be common [12]. Multi replicons are also common in the environment. A study found that the diversity of plasmids found in the environment was higher than in UTIs in the same area. *CTX-M-1*'s in the environment have been shown to be associated with IncI1 and IncN [43]. A report has suggested a direct link between wastewater treatment plants and ESBL-producing UTIs.

Sewage sludge used for agriculture may be one of the ways that ESBL-producing isolates enter the food chain [43]. It has been established that *CTX-M-1* harbouring E.coli in manure spread on fields can survive in the soil for at least a year. This demonstrates the capability of antibiotic resistant bacteria to survive under environmental conditions in the absence of antibiotic selection pressure [21].

A limitation of this study is that isolates were not categorised into source of isolate (community/hospital acquired). However, other studies suggest that the *CTX-M* family and ESBLs in general are associated with community-acquired infection [4,49]. In this study, we only collected ESBL-positive isolates. From this, we cannot determine the level of ESBL resistance as a whole. This study should be followed up regularly, to study the trends of antibiotic resistance in this area. More surveillance studies on animal and environmental isolates are needed, to fully understand the relationship between these and human infections.

We have reported similar results to what has been found in the rest of the UK and worldwide. *CTX-M*-harbouring Enterobacteriaceae is a major threat to patient care and healthcare costs. Genotypic prevalence studies are an important contribution to the understanding and prediction of antibiotic resistance evolution and antibiotic stewardship. Knowledge of plasmid types circulating in bacterial populations is vital to advancing a new prospective to control these plasmids, such as replicon-targeting compounds. A strategy to prevent the further dissemination of these plasmids needs to be implemented.

Combined, these findings highlight the importance of restricting antibiotics sales to only those with medical prescriptions and appropriate use, as many low and middle-income countries have not yet enforced policies that prevent widespread self-medication with antibiotics.

This study reinforces the One Health approach and underpins the importance of antibiotic stewardship and infection prevention schemes in humans, animals, food production and the environment in order to limit the spread of ESBL-harbouring Enterobacteriaceae, not only in the UK, but worldwide.

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Conflict of Interest

The authors declare no conflict of interest.

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